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Research Article

Genetic assessment of the introduced rats found on Lord Howe Island post rodent eradication, new invaders or remnant survivors?

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Abstract

The black rat (*Rattus rattus*) is one of the most prevalent invasive species globally, having significant impacts across ecosystems as a competitor, predator, and driver of disease for native biota. These impacts have been especially severe on islands. Rat eradication programmes as a conservation management intervention have been undertaken on many islands around the world, including on Australia's Lord Howe Island (LHI), located in the southwest Pacific, 600 km east of the Australian mainland. In 1918, the black rat was accidentally introduced to LHI, now recognised as a World Heritage-listed biodiverse subtropical island. The impact on LHI's unique biodiversity was catastrophic, with many endemic taxa becoming extinct or severely declining. A Rodent Eradication Programme (REP) for LHI was launched in early 2019, and by October 2019 detections of rodents had ceased. However, in April 2021, black rats were again detected in the settlement area of LHI. To determine if the rats detected on LHI post-REP were new invaders or remnant individuals, mitochondrial DNA and microsatellite analysis were carried out. Only a single identical CytB haplotype was detected in the pre- and post-REP LHI rats. The black rats sampled from New South Wales (NSW) on the east coast of the Australian mainland and southwest Pacific islands mostly had similar but consistently different CytB haplotypes to LHI rats, or highly divergent haplotypes. Microsatellite data demonstrated that the NSW mainland and LHI pre-REP rat populations were genetically differentiated, with little evidence of gene flow between them in either direction despite potential dispersal routes. The LHI post-REP rats had low genetic diversity, were highly interrelated, and appear to be descended from a small number of individuals. Genetic clustering analyses grouped the LHI post-REP rats with the LHI pre-REP population rather than the NSW mainland population. Thus, both the microsatellite and mtDNA results are consistent with the LHI post-REP rats being the descendants of a small number of the LHI pre-REP population and not new invaders from the NSW mainland population.

Key words: Cytochrome b, island, microsatellite, mtDNA, Pacific Ocean, *Rattus*, rodent eradication

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Introduction

Originating in south and southeast Asia, the black rat, *Rattus rattus* (also known as the ship rat, roof rat, or house rat), is one of the most successful human commensal organisms (Aplin et al. 2011) and an invasive species globally (Capizzi et al. 2014; Harper and Bunbury 2015). Black rats have generated

devastating environmental impacts, both historical and ongoing, especially on island biodiversity (Towns et al. 2006; Varnham 2010; Harper and Bunbury 2015), and rat eradication programmes are now commonly implemented as a form of environmental management on islands to halt species loss and restore ecosystems (Keitt et al. 2011; Capizzi et al. 2014; Russell and Holmes 2015). Pennisi (2024) cited 820 attempts of rodent eradication from 666 islands globally. Rodent eradication programmes are logistically complex and, even with meticulous planning and execution, can fail (Amos et al. 2016; Samaniego et al. 2021). Rodents either survive the eradication programme itself and re-establish a population, or new subsequent invaders take advantage of vacated habitat (Russell et al. 2010). Understanding the origin of rats identified after eradication efforts have been undertaken is important for understanding the efficacy of these initiatives and for effective management in their aftermath (Abdelkrim et al. 2007; Russell et al. 2010).

Population genetic tools have been employed as a vital complement to rodent eradication projects. Variation in the cytochrome B (CytB) gene has been widely studied in global black rat populations (Aplin et al. 2011; Lack et al. 2012; Conroy et al. 2013; Ramatla et al. 2019; Etougbétché et al. 2020; Thomson et al. 2022; Pimsai et al. in press). While this gene region cannot provide sufficient resolution to determine the specific origin of particular black rat populations, it nevertheless can place them in a global genetic context, which may provide clues as to their origins (Aplin et al. 2011). Globally, black rats comprise four well-differentiated mitochondrial DNA lineages (termed Lineages I–IV) that may represent incipient species (Aplin et al. 2011). Lineage II rats have sometimes been recognised as a distinct species, the tanezumi or oriental house rat, *Rattus tanezumi* (Musser and Carleton 2005; Aplin et al. 2011). Two of the four black rat lineages (I and II) have spread widely around the world, with Lineage I largely in association with European exploration and colonial expansion (Aplin et al. 2011). In a global study of black rat diversity, Aplin et al. (2011) detected Lineage I rats in eastern Australia, as well as in New Zealand, New Guinea, and other Pacific islands, although sampling was limited. Lineage II rats were also detected in New Guinea (Aplin et al. 2011). It is now known that Lineage I and II black rats readily interbreed when sympatric and that extensive introgression of alleles can occur (Chinen et al. 2005; Lack et al. 2012; Conroy et al. 2013). For this reason, we currently regard Lineage I and II rats as a single species: *Rattus rattus*, the black rat. Higher-resolution nuclear DNA (nDNA) markers distributed across the genome (e.g., microsatellites) are generally deployed to design and monitor eradication programmes. This helps to establish the dispersal capabilities of target species and the geographic dimensions to be considered (unit of eradication) (e.g., Abdelkrim et al. 2005; Fratini et al. 2020). This can also provide the resolution required to identify the population source of rats that may turn up post-eradication, i.e., whether they are remnant survivors (Abdelkrim et al. 2007; Griffiths et al. 2014; Amos et al. 2016; Capizzi et al. 2024) or new invasions or reinvasions from elsewhere (Abdelkrim et al. 2007; Russell et al. 2010; Savidge et al. 2012).

Black rats likely first arrived in Australia with Dutch explorers in the 17th century; however, it is thought that it was not until their arrival in eastern Australia via the fleet of British ships that founded a settlement at Sydney Cove in 1788 that black rats became firmly established on the continent (Banks et al. 2023). Black rats are now found across Australia, especially in urban areas

and forest habitats (Banks and Hughes 2012; Banks et al. 2023). They have also been recorded on at least 70 of Australia's offshore islands (Abbott and Burbidge 1995). One of these islands, Lord Howe Island (LHI), is a World Heritage-listed, 1455 ha volcanic remnant island in the Tasman Sea, located ~600 km off the New South Wales (NSW) coast. Originally uninhabited, the island was first settled in 1834 (Hutton 1991) and now has a population of around 350 people, with no more than 400 tourists allowed on the island at one time (Harper et al. 2020). Between 1910 and 1931, cargo and passengers arrived on LHI via the steamboat SS *Makambo* on its regular route between eastern Australia, including Sydney, and the islands of Melanesia. In June 1918 the SS *Makambo* ran aground on LHI for 9 days, during which black rats aboard the ship escaped onto LHI (McCulloch 1921; Hutton 1991).

The introduction of black rats to LHI was devastating to the island's unique biodiversity (McCulloch 1921; Wilkinson and Priddel 2011). Black rats were in plague proportions within 2 years of arrival and are implicated in the extinction of five endemic bird taxa (McCulloch 1921; Hutton 1991), as well as the extinction or decline of many other endemic species, including reptiles (Cogger 2004), land snails (Hyman and Köhler 2020), insects (Cassis et al. 2003; Priddel et al. 2003; Reid and Hutton 2024), and plants (Auld and Hutton 2004; Auld et al. 2010). As a response to these impacts, predation by black rats on Lord Howe Island was listed as a Key Threatening Process under both NSW and Australian federal legislation (DECC 2007). Eradicating rodents on LHI (both rats and introduced house mice, *Mus musculus*) was first proposed in the early 2000s; however, the complexities of delivering this eradication programme—one of the first rodent eradications attempted within and around a permanently settled island community—meant it was not until early 2019 that the Rodent Eradication Programme (REP) had sufficient community backing and support to be rolled out (LHIB 2019; Walsh et al. 2019; Harper et al. 2020). The REP deployed the rodenticide Brodifacoum via a combination of methods, including ground-based applications (e.g., bait stations and hand-broadcast) across 300 ha, including in the settlement, and aerial bait applications across a further 1200 ha, including forested areas (Harper et al. 2020). The REP was deemed successful, and the last rat was detected on LHI on 9 October 2019 (Harper et al. 2020). However, in April 2021 black rats were detected once again in the settlement area of LHI, the first time since October 2019. It was unknown if these 2021 individuals represented residual rats that had persisted in the LHI settlement area despite the REP and ongoing monitoring, or an incursion by recent new arrivals (most likely from traffic to the island from eastern mainland NSW), or potentially both. Pathways of possible invasion for rats onto LHI from eastern Australia include regular air services (from Sydney and the regional centre of Port Macquarie on the Hastings River in north-eastern NSW) or by sea. Possible sea routes include recreational sailors or the fortnightly barge service from Birdon, near Port Macquarie (LHIBS 2016) (this barge service previously ran from Goodwood Island in the Clarence River near Yamba further north along the NSW coast). The aims of this study were to combine mtDNA and nDNA analyses to determine the source of the LHI post-REP rats found on LHI in April 2021 and provide the Lord Howe Island Board (LHIB) with information to assist their management protocols and biosecurity monitoring on LHI to ensure the island stays rodent-free.

Methods

Study site

Lord Howe Island (31°33'S, 159°05'E) is a 1455 ha volcanic island located approximately 600 km east of the Australian mainland in the southwest Pacific. The island is about 12 km long and ranges from 1–2 km wide in the shape of a crescent (Miller and Mullette 1985; O'Dwyer et al. 2024). It has a subtropical climate with mild to warm summers and regular rainfall (annual average ~1600 mm) (Bureau of Meteorology 2025). The majority of the island is protected under a Permanent Park Preserve (similar to national park status), and the vegetation is predominantly oceanic rainforests and palm forests. Around 25% of the island has been cleared for the permanent settlement with 350 inhabitants and other land uses (e.g., grazing) (O'Dwyer et al. 2024). All the LHI black rats analysed in this study were collected from in and around the settlement area (Fig. 1).

Sample collection

A total of 130 black rats were sampled for this study (Fig. 1): 38 collected from LHI in 2021 (post-REP); 27 representing LHI pre-REP populations (n = 14

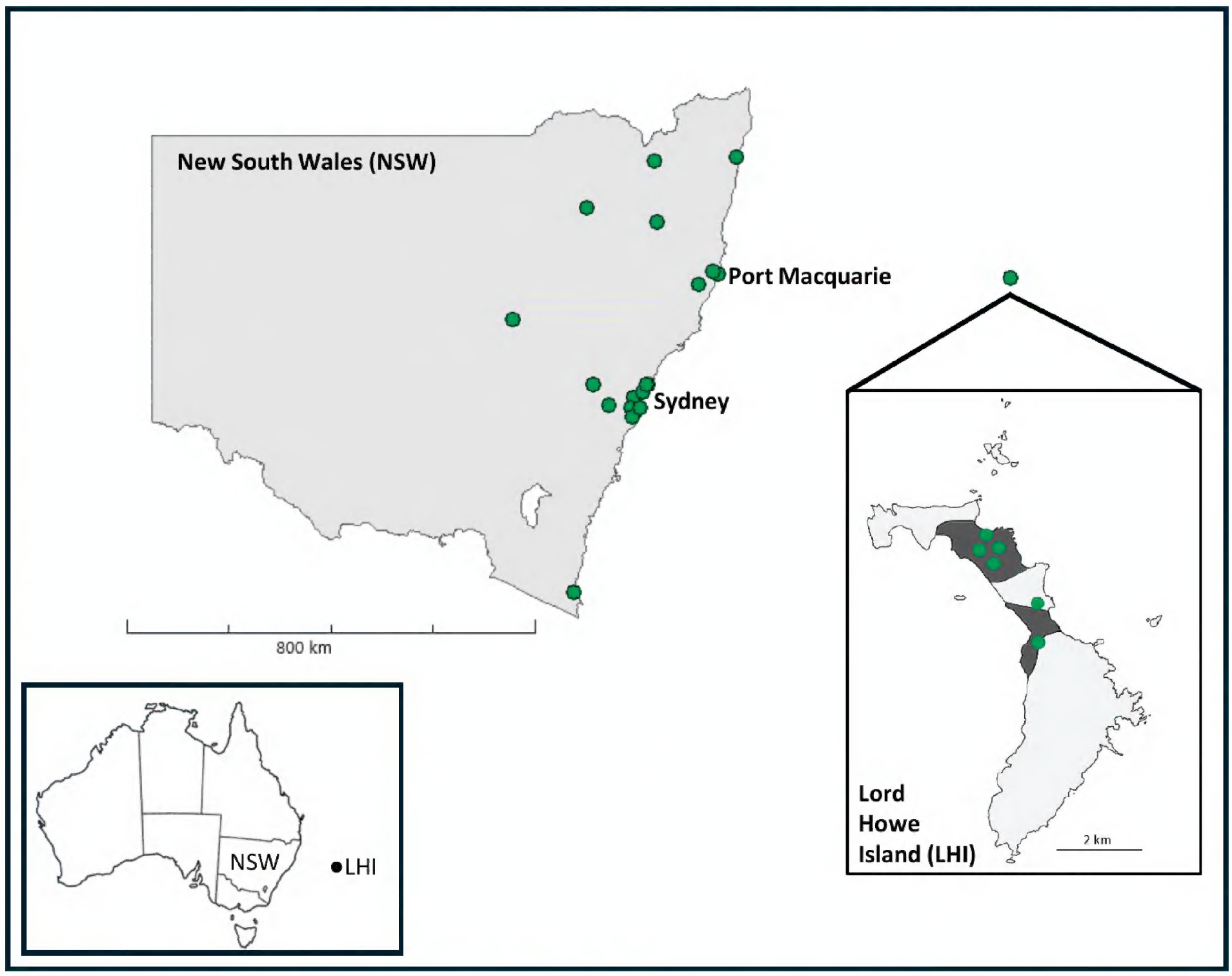

Figure 1. Geographic distribution of black rats sampled in New South Wales, Australia, for this study, with Lord Howe Island included in the larger box insert. All Lord Howe Island samples were collected in and around the settlement area on the island, which is shaded.

collected in 2003 and n = 13 collected in 2017); 60 from mainland NSW; and 5 from southwest Pacific islands, including Norfolk Island, Papua New Guinea, and Vanuatu. The LHI post-REP rats were collected by the LHIB; other samples were from the Australian Museum Mammalogy collection or were provided by NSW Department of Primary Industries (DPI), NSW National Parks and Wildlife Service (NPWS), and Taronga Zoo staff. Mainland samples were sourced throughout eastern NSW, from north of Glen Innes south to Eden and west to near Narrabri (Fig. 1). The most heavily sampled areas were those representing likely potential sources for rat incursions, including the Hastings River Valley (Birdon/Port Macquarie) (n = 18), greater Sydney (n = 16), and Goodwood Island on the Clarence River (n = 15). (See Suppl. material 1: table S1 for a list of individuals used in this study.) Due to varying sample quality, not all samples were included in all analyses.

DNA extraction and quantification

Genomic DNA was extracted from frozen tissue samples according to a high-salt method (Sunnucks and Hales 1996) or using the ISOLATE II Genomic DNA kit (Meridian Biosciences, Cincinnati, USA). DNA extracts were quantified using a NanoDrop spectrophotometer and Qubit fluorometer with the dsDNA Broad Range Assay Kit following standard protocols (Life Technologies, Carlsbad, USA).

Mitochondrial DNA amplification and analysis

To investigate samples for mtDNA variation, a 307 base pair (bp) region of the CytB gene was amplified using primers from Kocher et al. (1989): L14841 (5' AAAAAGCTTCCATCCAAC ATCTCAGCATGATGAAA 3') and H15149 (5' AAACTGCAGC CCCTCAGAATGAT ATTTGTCCTCA 3'). After the major mtDNA clades within the sampled rats had been identified (Fig. 2), a larger 927 bp region of CytB was amplified using primers from Aplin et al. (2011): L14724_Rattus (5' TGAYATGAAAAAYCATCGTTG 3') and H15915_Rattus (5' CATTTCAGGTTTACAAGA C 3') from a subset of samples that spanned the identified diversity (n = 66).

Polymerase chain reactions (PCRs) were carried out in 25 µl reactions using 100–1000 ng of genomic DNA, 1 × Reaction Buffer (MyTaq Reagent Buffer Red; Meridian Bioscience, Australia), 2 pmol primers, and MyTaq Red DNA polymerase (0.5 unit) (Meridian Bioscience, Australia). Negative control reactions were included in each PCR. Thermocycling was performed on an Eppendorf Mastercycler EpS (Eppendorf, Hamburg, Germany) under the following conditions; L14841/H15149—initial denaturation (94 °C for 3 min); 38 cycles of denaturation (94 °C for 20 s), annealing (55 °C for 40 s), and extension (72 °C for 40 s), followed by a final extension (5 min at 72 °C); L14724_Rattus/H15915_Rattus—initial denaturation (94 °C for 3 min); 1 cycle per degree—denaturation (94 °C for 50 s), annealing (touchdown 56–51 °C for 50 s in 1 °C decrements), and extension (72 °C for 60 s), followed by 38 cycles of denaturation (94 °C for 50 s), annealing (50 °C for 50 s), and extension (72 °C for 60 s), with a final extension (5 min at 72 °C). PCR products were cleaned using ExoSap-IT© (Applied Biosystems, Waltham, USA). Sequencing was performed on an AB 3730xl Sequencer at the Australian Genome Research Facility (AGRF) in Sydney.

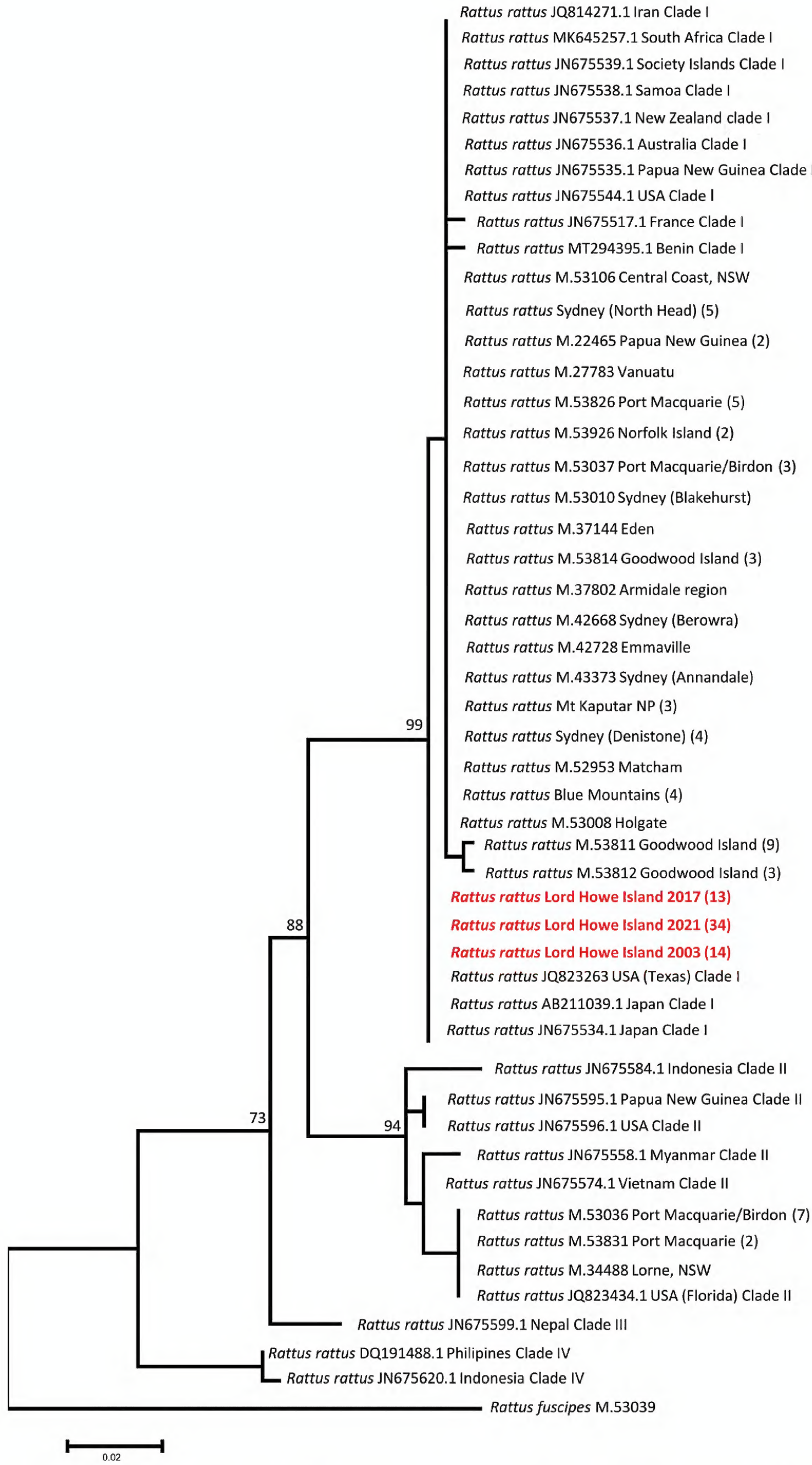
Rattus rattus JQ814271.1 Iran Clade I
Rattus rattus MK645257.1 South Africa Clade I
Rattus rattus JN675539.1 Society Islands Clade I
Rattus rattus JN675538.1 Samoa Clade I
Rattus rattus JN675537.1 New Zealand clade I
Rattus rattus JN675536.1 Australia Clade I
Rattus rattus JN675535.1 Papua New Guinea Clade I
Rattus rattus JN675544.1 USA Clade I
Rattus rattus JN675517.1 France Clade I
Rattus rattus MT294395.1 Benin Clade I
Rattus rattus M.53106 Central Coast, NSW
Rattus rattus Sydney (North Head) (5)
Rattus rattus M.22465 Papua New Guinea (2)
Rattus rattus M.27783 Vanuatu
Rattus rattus M.53826 Port Macquarie (5)
Rattus rattus M.53926 Norfolk Island (2)
Rattus rattus M.53037 Port Macquarie/Birdon (3)
Rattus rattus M.53010 Sydney (Blakehurst)
Rattus rattus M.37144 Eden
Rattus rattus M.53814 Goodwood Island (3)
Rattus rattus M.37802 Armidale region
Rattus rattus M.42668 Sydney (Berowra)
Rattus rattus M.42728 Emmaville
Rattus rattus M.43373 Sydney (Annandale)
Rattus rattus Mt Kaputar NP (3)
99
Rattus rattus Sydney (Denistone) (4)
Rattus rattus M.52953 Matcham
Rattus rattus Blue Mountains (4)
Rattus rattus M.53008 Holgate
Rattus rattus M.53811 Goodwood Island (9)
Rattus rattus M.53812 Goodwood Island (3)
Rattus rattus Lord Howe Island 2017 (13)
Rattus rattus Lord Howe Island 2021 (34)
Rattus rattus Lord Howe Island 2003 (14)
Rattus rattus JQ823263 USA (Texas) Clade I
Rattus rattus AB211039.1 Japan Clade I
Rattus rattus JN675534.1 Japan Clade I
88
Rattus rattus JN675584.1 Indonesia Clade II
Rattus rattus JN675595.1 Papua New Guinea Clade II
Rattus rattus JN675596.1 USA Clade II
73
94
Rattus rattus JN675558.1 Myanmar Clade II
Rattus rattus JN675574.1 Vietnam Clade II
Rattus rattus M.53036 Port Macquarie/Birdon (7)
Rattus rattus M.53831 Port Macquarie (2)
Rattus rattus M.34488 Lorne, NSW
Rattus rattus JQ823434.1 USA (Florida) Clade II
Rattus rattus JN675599.1 Nepal Clade III
Rattus rattus DQ191488.1 Philipines Clade IV
Rattus rattus JN675620.1 Indonesia Clade IV
Rattus fuscipes M.53039
0.02

Figure 2. Relationship amongst CytB haplotypes (307 bp) from LHI, eastern Australian, and global black rat data from GenBank.

Sequences were checked and edited using Sequencher v5.2.4 (Gene Codes Corporation, Ann Arbor, USA). Sequence alignment was carried out in MEGA v6 with comparison to published black rat CytB sequences from GenBank (Aplin et al. 2011; Lack et al. 2012; Conroy et al. 2013; Ramatla et al. 2019; Etougbétché et al. 2020). To estimate the phylogenetic relationships among haplotypes, an appropriate model for DNA substitution was determined using MEGA v6 (Nei and Kumar 2000; Tamura et al. 2013). Maximum likelihood trees were also constructed in MEGA v6 with 1000 bootstrap replicates, using the T92+G (Tamura 1992) (307 bp) and HKY+G (Hasegawa et al. 1985) (927 bp) models.

Nuclear DNA (microsatellite) analysis

Genotyping

A total of 148 samples, representing 130 individual rats, were genotyped over two different genotyping runs, with 18 samples run in duplicate or triplicate for quality control or to improve genotyping data between runs. Genotyping was carried out by AGRF in Melbourne using an Applied Biosystems AB 3730xl capillary analyser. The initial genotyping run was carried out using 15 microsatellite loci described by Loiseau et al. (2008) and Louys et al. (2020) (see Suppl. material 1: table S2 for details of loci). These microsatellite loci were multiplexed and labelled with fluorescent dye based on expected size ranges, and genotypes were scored using GENEMAPPER 4.1 (Applied Biosystems, Waltham, USA). The second run was carried out using the 12 loci identified as informative in the previous run (Suppl. material 1: table S2).

Data analysis

Standard population and diversity analyses

Data quality was assessed using GenAlEx v6.5 (Peakall and Smouse 2012). Conformance to Hardy–Weinberg equilibrium (HWE) expectations and standard population genetic diversity indices and relatedness (Queller and Goodnight 1989) were calculated for the whole data set using GenAlEx v6.5 (Peakall and Smouse 2012).

Genetic differentiation between two potential source populations

Potential genetic differentiation was investigated using four different methods in GenAlEx v6.5 (Peakall and Smouse 2012). This included calculation of F_{ST}; identification of unique alleles; principal coordinate analyses (PCoA) of allele frequencies; and Bayesian clustering analysis using STRUCTURE v2.3.4 (Pritchard et al. 2000). STRUCTURE analyses were run without prior population-of-origin information, uncorrelated gene frequencies, and allowing admixture. Markov chain Monte Carlo simulations were carried out with 10 replicates for values of K (genetic clusters) from 1–5, with 1,000,000 iterations after a 100,000 burn-in. Maximum delta log-likelihood (△K) (Evanno et al. 2005) and the maximum posterior probability (L(K)) (Pritchard et al. 2000) were used to identify the optimal number of genetic clusters present in the data using Cluster Markov Packager Across K (CLUMPAK) v1.1 (Kopelman et al. 2015) and STRUCTURE HARVESTER (Earl and Von Holdt 2012). Structure plots were visualised using STRUCTURE PLOT v2 (Ramasamy et al. 2014).

Table 1. Pairwise relatedness values within sampled populations of black rat, *Rattus rattus*.

Population		Lynch and Ritland (1999) estimator	Queller and Goodnight (1989) estimator
Lord Howe Island pre-REP	**Range**	–0.080–0.456	–0.276–0.866
	Mean	0.134	0.322
	SE	0.006	0.011
Lord Howe Island post-REP	**Range**	–0.027–0.500	0.108–1.00
	Mean	0.320	0.737
	SE	0.003	0.005
Goodwood Island	**Range**	–0.049–0.396	–0.295–0.725
	Mean	0.134	0.263
	SE	0.09	0.021
Eastern NSW	**Range**	–0.102–0.441	–0.423–0.891
	Mean	0.035	0.080
	SE	0.002	0.007

Testing the genetic affinities of the LHI post-REP individuals

Most data analysis methods in population genetics assume that individuals sampled from the population being investigated are unrelated (Frankham et al. 2010, 2017). The LHI post-REP rat population posed a challenge in this regard, as all appeared highly related (Table 1). We therefore limited testing of genetic affinity of the LHI post-REP individuals to a dataset from which subadults, juveniles, and embryos were removed, leaving six LHI post-REP adult individuals (M.53026, M.53073, M.53074, M.53076, M.53077, M.53079). The genetic affinities of these LHI post-REP individuals were tested using three methods: direct comparison of alleles, including unique alleles; Bayesian clustering analysis using STRUCTURE v2.3.4 (Pritchard et al. 2000); and principal coordinate analyses (PCoA) of allele frequencies using GenAlEx v6.5 (Peakall and Smouse 2012).

Parameters for the STRUCTURE analysis were as described above, but with 10 replicates for values of K (genetic clusters) from 1–6, with 1,000,000 iterations after a 100,000 burn-in. Maximum delta log-likelihood (△K) (Evanno et al. 2005) and the maximum posterior probability (L(K)) (Pritchard et al. 2000) were used to identify the optimal number of genetic clusters present in the data using CLUMPAK v1.1 (Kopelman et al. 2015) and STRUCTURE HARVESTER (Earl and Von Holdt 2012).

Seven separate PCoAs of allele frequencies were carried out in GenAlEx v6.5 (Peakall and Smouse 2012); the first containing all six LHI post-REP adult rats and then another six in which each analysis included a different single 2021 LHI post-REP individual. This was done to avoid the tendency of highly genetically similar individuals biasing the outcome by clustering together as distinct from other samples from their population (Ewart et al. 2021).

Results

Mitochondrial DNA analysis

A total of 307 bp of CytB was amplified from 125 individual rats (GenBank accession numbers: PV646295–PV646420, Suppl. material 1: table S1). All rats from LHI (pre- and post-REP) shared the same Lineage I haplotype (n = 62) (Fig. 2).

The LHI haplotype was consistently different from the haplotypes found in the rats sampled from mainland NSW. Most mainland NSW black rats (n = 48) shared a Lineage I haplotype, including all rats from greater Sydney (n = 16) and some from Port Macquarie (n = 8) (Fig. 2), although 10 mainland NSW rats, all sampled from Port Macquarie and surrounds, had a divergent Lineage II haplotype (Fig. 2).

A total of 927 bp of CytB was amplified from 66 individual rats that spanned the haplotype diversity detected by the 307 bp fragment (Fig. 2) (GenBank accession numbers: PX001639–PX001704, Suppl. material 1: table S1). Although this dataset contained substantially more sequence data and some additional sequence variation was detected, the same basic pattern of relationships was resolved (Suppl. material 1: fig. S1). All sequenced rats from LHI (pre- and post-REP) shared the same identical haplotype. This haplotype differed from the haplotypes found in rats sampled from mainland NSW and southwest Pacific islands. The majority of rats from mainland NSW shared similar haplotypes, although seven individuals sampled from the Port Macquarie area had divergent Lineage II haplotypes (Suppl. material 1: fig. S1).

Microsatellite analysis

Of the 130 individual rats genotyped, 123 individuals passed quality checks to make up the final dataset for analyses. All individuals that were genotyped in duplicate/triplicate produced identical genotypes between runs. Samples with missing data at more than 2 loci (~16% missing data) were removed from the final analyses. Three loci (Rr114, Rr25, and Rr93) were removed from the panels between genotyping runs due to high rates of missing data.

All 12 loci were polymorphic across the samples, detecting between 3 and 14 alleles per locus (Suppl. material 1: table S3). However, locus Rr17 was monomorphic in the LHI pre-REP samples, and loci Rr14, Rr17, Rr24, Rr26, and Rr28 were monomorphic in the LHI post-REP samples (Suppl. material 1: table S3). All loci in all sampled populations conformed to HWE expectations except for four loci (Rr6, Rr8, Rr15, and Rr26) for the mainland NSW samples. This is most likely due to the widespread geographic sampling in eastern NSW, which is likely to include individuals from different populations, and the presence of rats of Lineage II genetic background, which would violate HWE assumptions.

Standard population and diversity analyses

Detected levels of genetic diversity (N_a and H_e) were high in the mainland NSW population, moderate in the LHI pre-REP population, and low in the LHI post-REP population (Table S1). For example, the mean number of alleles per locus (N_a) is ~50% lower in the LHI pre-REP population than on the mainland and ~50% lower in the LHI post-REP population compared to the LHI pre-REP population (Table 2). This low diversity in the LHI post-REP sample is due to 5 of the 12 loci being monomorphic and 6 of the remaining 7 loci only having a maximum of 2 alleles (Suppl. material 1: table S3), which reduces the available information content for analysis. Individuals within the LHI post-REP sample were highly inter-related (Table 1), showing a mean relatedness (Queller and Goodnight 1989) value of 0.737, with values ranging from 0.108–1.00 (Table 1), indicating that most individuals are likely to be first-degree relatives.

Table 2. Standard population genetic diversity indices for sampled black rat, *Rattus rattus* populations.

Population		*N*	*Na*	*Ne*	*Ho*	*He*	*F*
LHI pre-REP (2003 and 2017)	**Mean**	26.25	3.08	2.07	0.43	0.43	0.00
	SE	0.41	0.45	0.24	0.07	0.07	0.06
LHI post-REP (2021)	**Mean**	35.00	1.67	1.38	0.24	0.22	-0.11
	SE	0.62	0.19	0.11	0.07	0.06	0.04
Eastern NSW	**Mean**	53.67	6.83	3.42	0.48	0.60	0.14
	SE	1.22	0.89	0.51	0.06	0.08	0.05

N = mean sample size, *Na* = mean number of alleles per locus, *Ne* = number of effective alleles, *Ho* = observed heterozygosity, *He* = expected heterozygosity, *F* = Fixation index.

Genetic differentiation between the potential source populations

All four approaches detected genetic differentiation between the LHI pre-REP and mainland NSW populations. The estimated F_{ST} value between them was 0.10, and there were six unique alleles (16%) present in the LHI pre-REP population and 49 unique alleles (61%) in the eastern mainland NSW population (Suppl. material 1: table S3).

Both the STRUCTURE and PCoA analyses resolved two clusters, which corresponded to samples from LHI pre-REP and mainland NSW (Suppl. material 1: figs S2, S3). In both analyses, within the LHI pre-REP cluster, rats sampled in 2003 and 2017 were not differentiated (Suppl. material 1: figs S2, S3).

The Bayesian clustering analysis using STRUCTURE identified 2 (maximum ΔK) or 3 (maximum L(K)) genetic groupings within the analysed samples (Suppl. material 1: figs S2, S4). For K = 2, the two genetic clusters consisted firstly of rats sampled from LHI (pre-REP) and secondly of all sampled mainland NSW rats (Suppl. material 1: fig. S2). At K = 3, the LHI pre-REP rats continued to cluster together, while some additional geographic sub-structuring was identified within the mainland NSW rats, primarily separating out the samples from Goodwood Island (Suppl. material 1: fig. S2). There was little evidence of gene flow, in either direction, between the LHI pre-REP population and the NSW mainland population. At K = 2, six mainland individuals showed a proportion of membership (> 0.10) to the LHI cluster in the STRUCTURE analysis (Suppl. material 1: fig. S2). These six were from scattered sampling sites across mainland NSW, including greater Sydney (Berowra, Annandale, Blue Mountains) and northern NSW (Emmaville, Birdon, Goodwood Island), although proportions greater than 0.5 were only found in samples from Emmaville and the Blue Mountains. At K = 3, as additional structure was identified within the mainland population, the proportion of membership to the LHI cluster for mainland individuals was reduced (Suppl. material 1: fig. S2).

In the PCoA, two clusters were resolved (LHI pre-REP and mainland) with 13.95% of the variation explained by PCo1 and 8.86% explained by PCo2 (Suppl. material 1: fig. S3).

Testing the genetic affinities of the LHI post-REP individuals

The sampled LHI post-REP individuals had low genetic diversity and were all very similar genetically. Overall, the allelic profile of the LHI post-REP sample is very similar to the LHI pre-REP sample. Only 20 alleles were present in the

LHI post-REP population, of which 19 (95%) were shared with the LHI pre-REP population, including the presence of 3 unique LHI alleles (Suppl. material 1: table S3). Compared to the NSW mainland population, the LHI post-REP population shared 16 (80%) alleles, but no unique mainland alleles were present (Suppl. material 1: table S3). For comparison, the LHI pre-REP and NSW mainland populations share 31 (39%) alleles (Suppl. material 1: table S3); these are mostly high-frequency alleles that are likely to be widespread in rat populations. Thus, the alleles present in the LHI post-REP sample are largely a subset of those present in the LHI pre-REP sample rather than from the NSW mainland.

The Bayesian clustering analysis using STRUCTURE identified 2 (maximum ΔK) or 3 (maximum L(K)) genetic groupings within the analysed samples (Fig. 3, Suppl. material 1: fig. S5). For K = 2, the two genetic clusters that were identified consisted firstly of rats sampled from LHI (both pre- and post-REP) and secondly of all sampled mainland NSW rats (Fig. 3). At K = 3, the pre- and post-REP rats from LHI continued to be clustered together, while some additional genetic structure was identified within the mainland NSW rats, mainly involving the separation of samples from Goodwood Island (Fig. 3).

The initial results of the PCoA resolved two broad clusters of individuals, corresponding to the NSW mainland rats and the LHI rats (both pre-REP and post-REP) (Fig. 4A). A total of 15.09% of the variation was explained by PCo1, and 8.55% was explained by PCo2 (Fig. 4A). The six highly related LHI post-REP adults were clustered together on the edge of the LHI pre-REP cluster (Fig. 4A). To test whether this grouping of the six LHI post-REP rats was a product of their high relatedness (Ewart et al. 2021), we also re-analysed each separately. When each of the six LHI post-REP rats was assessed individually, their position altered, with all now clustering within the LHI pre-REP population (Fig. 4B).

Discussion

This study aimed to determine if the rats detected on LHI in April 2021 were remnants of the LHI pre-REP population or new invaders that had arrived on LHI post-eradication efforts from the NSW mainland. Both the microsatellite and mtDNA data demonstrated that the LHI pre-REP and sampled mainland NSW black rat populations could be genetically differentiated. Additionally, these data showed that rats sampled on LHI in April 2021 had genetic affinity to the LHI pre-REP rat population. Therefore, it could be concluded that these individuals were remnant LHI pre-REP rats.

Mitochondrial DNA data

All sampled rats from LHI (both pre- and post-REP) shared the same identical mtDNA CytB haplotype. The single haplotype detected in LHI rats is a Lineage I haplotype that is identical or very similar to published haplotypes from black rats sampled in Hokkaido, Japan, and Texas, USA (Chinen et al. 2005; Aplin et al. 2011; Lack et al. 2012) (Fig. 2). This haplotype has not yet been detected in rat populations sampled from mainland Australia, Melanesia, or the southwest Pacific. The LHI haplotype differed consistently from the haplotypes found in the rats sampled from eastern mainland NSW, most (48/58) of which shared the same or very similar haplotypes previously identified as Lineage I haplotypes from rats sampled in eastern

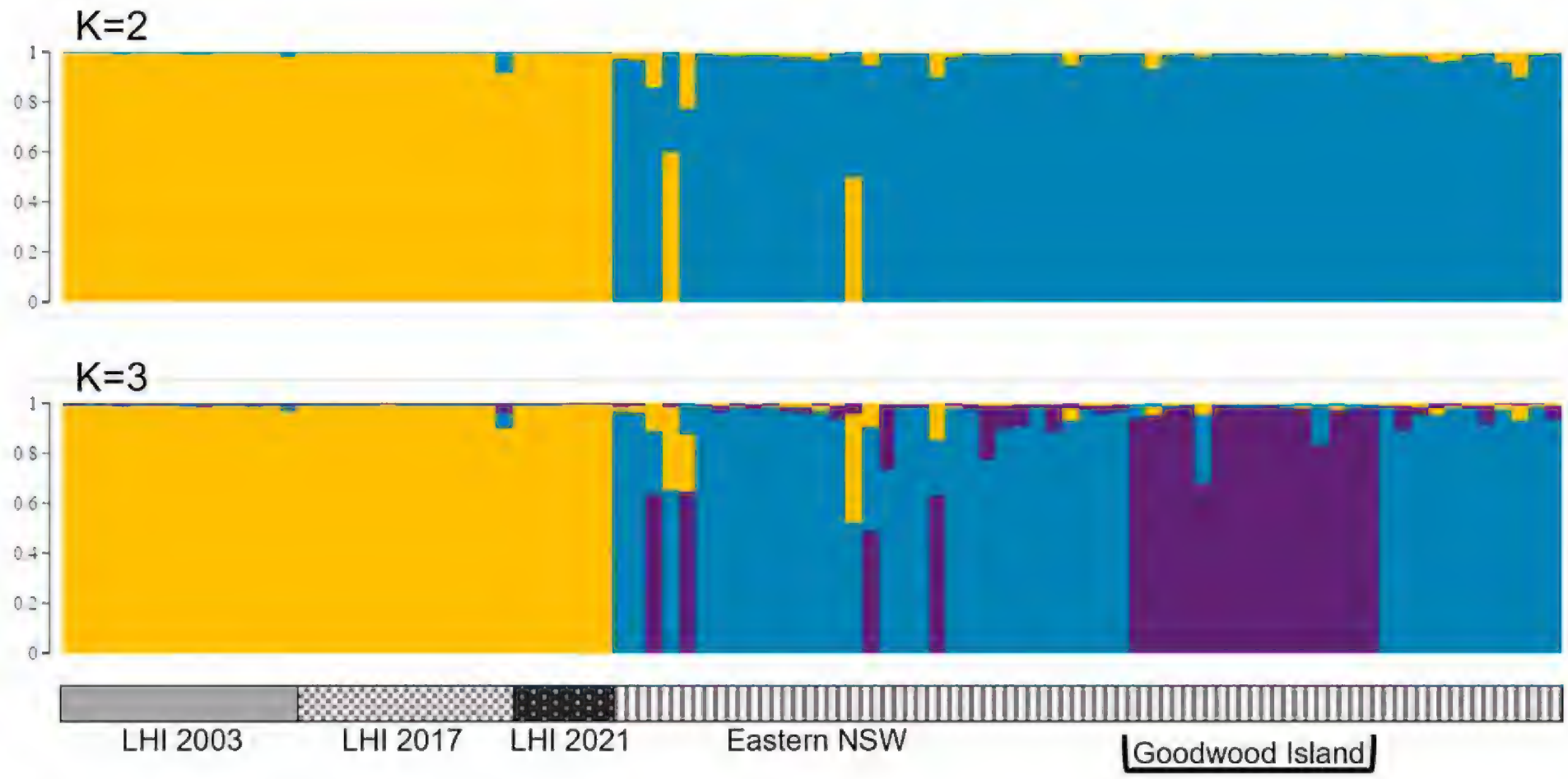

Figure 3. Results of Bayesian clustering analysis for black rats using STRUCTURE for LHI pre-REP, LHI post-REP, and mainland NSW rats.

Australia, New Zealand, Papua New Guinea, Samoa, the Society Islands, South Africa, western Africa, Iran, France, and the USA (Aplin et al. 2011; Conroy et al. 2013; Ramatla et al. 2019; Etougbétché et al. 2020) (Fig. 2). Lineage I haplotypes were also identified in rat samples sequenced from Norfolk Island, Papua New Guinea, and Vanuatu as part of this study (Fig. 2). Ten rats sampled from the Port Macquarie area were found to have a divergent Lineage II haplotype that was identical or very similar to published haplotypes from Florida, USA, and southeast Asia (Aplin et al. 2011; Lack et al. 2012) (Fig. 2). The presence of black rats with Lineage I and Lineage II (the tanezumi rat lineage) haplotypes in eastern Australia is consistent with multiple introductions of black rats into eastern Australia since European settlement.

Microsatellite data

As expected, detected levels of genetic diversity (N_a and H_e) were highest in the large and widespread NSW mainland population and substantially less in the insular LHI pre-REP population (Table 2). It is common for island populations to show lower genetic diversity due to small population size, limited gene flow, and frequent bottlenecks at the time of population establishment (Frankham 1997, 1998). Diversity was lowest in the LHI post-REP population, with a high proportion of loci either monomorphic or having only 2 alleles (Suppl. material 1: table S3). This pattern of diversity in the LHI post-REP sample is typical of a population that is derived from a very small number of individuals (possibly as low as n = 2) and/or has spent multiple generations at low population size (Frankham et al. 2017). The sampled LHI post-REP individuals also showed high levels of pairwise inter-relatedness (Table 1), which is consistent with the above scenario.

Using the microsatellite data, all four approaches (F_{ST}, unique alleles, STRUCTURE, and PCoA) detected genetic differentiation between the LHI pre-REP and mainland NSW populations.

Black rats have been present on LHI for over 100 years and in eastern mainland NSW for over 200 years. LHI's geographic isolation has likely protected it from

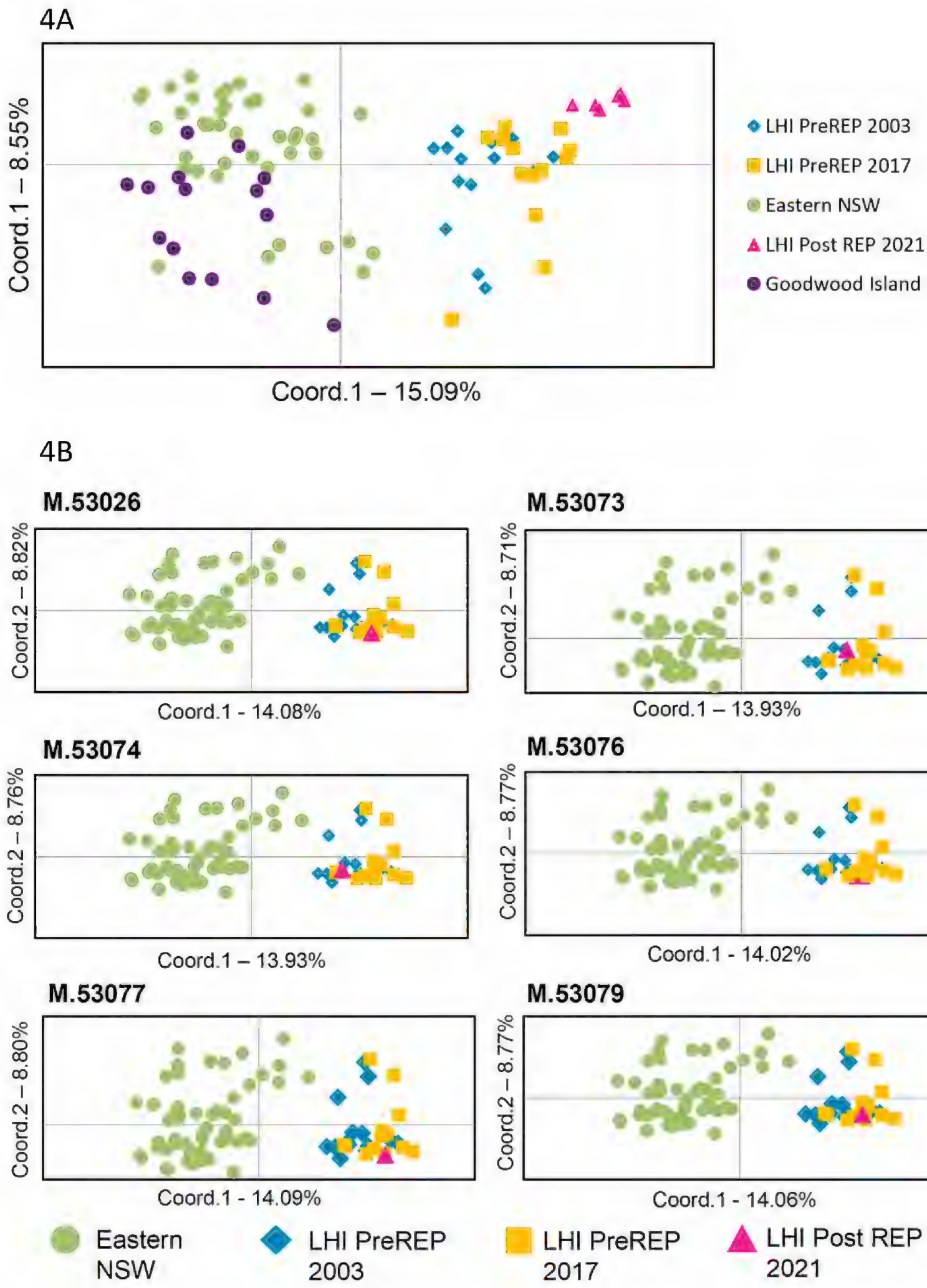

Figure 4. PCoA for LHI pre-REP, LHI post-REP, and mainland NSW black rats showing the clustering of all six post-REP adults **(4A)**, and then **(4B)** each adult post-REP rat individually (with Goodwood Island included in the eastern NSW samples).

the natural arrival of black rats (Capizzi et al. 2015), although the regular movement of people and goods still provided opportunities for human-mediated genetic exchange. Despite this, even with sampling efforts concentrated at the current and past mainland barge ports involved in transport to LHI (Birdon and Goodwood Island), there was surprisingly little evidence of gene flow between these two populations in either direction (Suppl. material 1: figs S2, S3). This is consistent with the idea of incumbent advantage, suggesting the LHI pre-REP population was so well established that new migrants could not establish or contribute their alleles to the resident LHI population (Granjon and Cheylan 1989; Abdelkrim et

al. 2007). This clear genetic differentiation between LHI pre-REP and the NSW mainland provided the resolution required to confidently distinguish between the two most likely origin scenarios for the 2021 LHI post-REP rats (i.e., LHI REP remnants versus new invaders from the NSW mainland).

The genetic affinities of the LHI post-REP rats

Analysis of both the mtDNA and microsatellite data (nDNA) rules out sampled rat populations in mainland NSW as the source of the LHI post-REP rats. Instead, both the mtDNA and microsatellite data are consistent with LHI post-REP rats being the descendants of the LHI pre-REP population. Identifying remnant survivors is a common finding when genetically evaluating rats found post-eradication (Abdelkrim et al. 2007; Griffiths et al. 2014; Amos et al. 2016; Capizzi et al. 2024). We cannot yet determine the original source population of the rats introduced to LHI in 1918; additional and more comprehensive data on black rat genetic variation globally will be necessary to understand this more completely. However, despite mainland Australia being identified as one of the source origins for black rats that have established on other offshore Australian islands, including Christmas Island and the Cocos–Keeling Islands (Thomson et al. 2022), based on the data available for this study, the LHI population does not appear to have originated from the east coast of Australia.

With respect to the mtDNA results, all the rats sampled on LHI (both pre- and post-REP) shared a single identical CytB haplotype, distinct from mainland NSW rats; this haplotype is not common globally but has been detected from widely scattered locations in Asia and North America (Chinen et al. 2005; Aplin et al. 2011; Lack et al. 2012). In relation to the nDNA analysis, microsatellite alleles present in the LHI post-REP rats are almost entirely a subset of those detected in the LHI pre-REP population, including the presence of some unique LHI alleles. Both these genetic patterns in the mtDNA (Savidge et al. 2012) and nDNA (Abdelkrim et al. 2007; Griffiths et al. 2014) have previously been identified in studies where rats have survived the initial eradication. The presence of a single unique allele in the LHI post-REP sample (i.e., not detected in either the LHI pre-REP or mainland sample) is likely an artefact of the limited sampling available for the LHI pre-REP population. This consists of 14 genotyped rats sampled on LHI in 2003 and 13 sampled in 2017. If considered separately, both these samples contain some alleles that are not present in the other sample (5 alleles in 2003; 3 alleles in 2017; Suppl. material 1: table S3), so it is conceivable that additional alleles were present in the LHI pre-REP population that were not detected in our limited sampling. In addition, genetic clustering and assignment analyses group the LHI post-REP rats with the LHI pre-REP population rather than with the NSW mainland. The fact that the STRUCTURE analysis could also separate the rats sampled from Goodwood Island (Fig. 3) from other mainland NSW populations further emphasises the genetic affinity of the LHI pre-REP and LHI post-REP rats.

The genetic diversity of LHI post-REP rats, both N_a (mean number of alleles per locus) and the expected heterozygosity (H_e), was substantially lower (by ~50%) compared to the LHI pre-REP population (Table 2). This suggests that the LHI post-REP population experienced a bottleneck more prolonged than a one-off reduction to a single pair, which is predicted to reduce H_e by only 25% (Frankham et al. 2010). In intense bottlenecks, the loss of allelic diversity (N_a) is expected to be more rapid than the decline in H_e (England et al. 2003), but this was not observed in the current situation where both parameters have declined by ~50% (Table 2).

Context and success of the LHI REP

One major difference between most island rat eradication programmes and the LHI REP is that LHI is an inhabited island. It is the largest of the inhabited islands that have been targeted for rodent eradications to date (Capizzi et al. 2024). While both the microsatellite and mtDNA data were consistent with LHI post-REP rats being the descendants of a very small number of the LHI pre-REP rats, the 18-month gap between the last rat detection during the REP in October 2019 and their reappearance in April 2021 does remain difficult to reconcile with an understanding of rat biology. One eradication programme that saw a similar time frame between initial eradication 'success' and the re-detection of remnant rats was on the island of Ventotene, Italy (Capizzi et al. 2024). Similar to LHI, Ventotene has a history of human settlement and development. The lag in detection of remnant rats in both of these REPs required a small number of pre-eradication rats persisting at low and essentially undetectable density for multiple generations in locations that had until recently supported large, continuously breeding populations. On LHI this occurred despite ongoing intensive monitoring (LHIB 2009; Harper and Bunbury 2015; Harper et al. 2020). Given the high fecundity, short generation time, and capacity for rapid population increase in favourable conditions for commensal rodents (Harper and Bunbury 2015), this seems unusual unless the population was continuing to be limited.

As widely acknowledged and discussed (LHIB 2009, 2019; Wilkinson and Priddel 2011; Walsh et al. 2019), the presence of 350 island residents and houses and associated infrastructure on LHI made the 2019 LHI REP a complex initiative, as did the diversity of the residents' views, opinions, and concerns. However, considerable thought, effort, lengthy consultations, and innovative and adaptive planning and management were undertaken to take these complex circumstances into account during the REP (Wilkinson and Priddel 2011; LHIB 2009, 2019; Walsh et al. 2019; Harper et al. 2020). When the rats reappeared on LHI in 2021, they did so in the settlement areas of the island, reinforcing the complexity that the presence of humans and their infrastructure causes for eradication programmes (Capizzi et al. 2024).

Once rats were detected on LHI in April 2021, a rapid and comprehensive response was launched to eradicate any remaining individuals. As of this publication, the last rat detected on LHI was in August 2021, and an upgraded REP and biosecurity programme was implemented using a range of rodent monitoring devices, including chew cards, wax tags, tracking tunnels, kill traps, detection dogs, and camera traps at high-risk reinvasion points and across the island to ensure LHI stays rat-free. Five years in, the native fauna and flora of LHI are already showing significant signs of recovery (LHIB 2023; O'Dwyer et al. 2023; Reid and Hutton 2024; O'Dwyer et al. 2025), demonstrating the ongoing success of this complex project.

Conclusion

Analyses of both the mtDNA and microsatellite data generated as part of this study demonstrate that the LHI post-REP rats detected in 2021 were remnants of the LHI pre-REP population. The use of genetic tools to monitor the success of eradication projects is not new (Abdelkrim et al. 2007; Russell et al. 2010; Amos et al. 2016), but studies such as this one demonstrate the need to include and properly resource genetic monitoring, including collection and archiving of samples prior to eradication initiatives, as part of eradication programmes and subsequent and

ongoing monitoring efforts. Fortunately, in this case, LHI pre-REP rats were available in the Australian Museum collection, collected as part of unrelated research carried out on LHI in 2003 and 2017, allowing for the origin of the April 2021 LHI post-REP rats to be determined.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Author contributions

Conceptualization: GJF, MDBE, KMH; Data curation: GJF, MDBE; Investigation: GJF; Formal analysis: GJF, MDBE; Writing - original draft: GJF, MDBE; Writing - review and editing: GJF, MDBE, KMH; Project administration; GJF, MDBE, KMH.

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Data availability

Data that support the findings of this study, including GenBank accession numbers, are available in the main text and supplementary data.

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Supplementary material 1

Suppl. tables and figures

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